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Holotransferrin Enhances Selective Anticancer Activity of Artemisinin against Human Hepatocellular Carcinoma Cells

Xiao-rong DENG (邓小荣)[1†#], Zhao-xia LIU (刘朝霞)[2†], Feng LIU (刘 峰)[3†], Lei PAN (潘 雷)[4], He-ping YU (余和平)[5#], Jin-ping JIANG (姜进平)[3], Jian-jun ZHANG (张建军)[1], Li LIU (刘 立)[1], Jun YU (喻 军)[1]
[1]*Department of General Surgery,* [2]*Department of Obstetrics and Gynecology, Nanshan Affiliated Hospital of Guangdong Medical College, Shenzhen 518052, China*
[3]*Department of General Surgery, First Affiliated Hospital of Nanchang University, Nanchang 330006, China*
[4]*Department of General Surgery, Qihe People's Hospital, Qihe 251100, China*
[5]*Department of General Surgery, Wuhan Pu'ai Hospital, Wuhan 430033, China*

Summary: Artemisinin, also termed *qinghaosu*, is extracted from the traditional Chinese medicine *artemesia annua L.* (the blue-green herb) in the early 1970s, which has been confirmed for effectively treating malaria. Additionally, emerging data prove that artemisinin exhibits anti-cancer effects against many types of cancers such as leukemia, melanoma, etc. Artemisinin becomes cytotoxic in the presence of ferrous iron. Since iron influx is high in cancer cells, artemisinin and its analogs selectively kill cancer cells with increased intracellular iron concentrations. This study is aimed to investigate the selective inhibitory effects of artemisinin on SMMC-7721 cells *in vitro* and determine the effect of holotransferrin, which increases the concentration of ferrous iron in cancer cells, combined with artemisinin on the anticancer activity. MTT assay was used for assessing the proliferation of SMMC-7721 cells treated with artemisinin. The induction of apoptosis and inhibition of colony formation in SMMC-7721 cells treated with artemisinin were determined by TdT-mediated dUTP nick end labeling (TUNEL) and colony formation assay, respectively. The results showed that artemisinin at various concentrations significantly inhibited growth, colony formation and cell viability of SMMC-7721 cells (P<0.05), likely due to induction of apoptosis of SMMC-7721 cells. Of interest, it was found that incubation of artemisinin combined with holotransferrin sensitized the growth inhibitory effect of artemisinin on SMMC-7721 cells (P<0.01). Our data suggest that treatment with artemisinin leads to inhibition of viability and proliferation, and apoptosis of SMMC-7721 cells. Furthermore, we observed that holotransferrin significantly enhanced the anti-cancer activity of artemisinin. This study may provide a potential therapeutic choice for liver cancer.
Key words: human hepatocellular carcinoma SMMC-7721 cells; artemisinin; holotransferrin; cell growth; colony formation; apoptosis

Hepatocellular carcinoma (HCC) is one of the most aggressive malignancies commonly seen in China. The treatment of HCC mainly depends on surgical resection. However, the rate of resection in HCC patients remains low[1]. In the meantime, the recurrence is a challenge to which surgeons have to face. Then, other non-surgical alternatives for the treatment of HCC are necessary and important. Among them, developing effective small molecular compounds from traditional Chinese medicine (TCM) has raised a large number of promising candidates, such as artemisinin.

Artemesia annua L. (*qinghao*) has been used for centuries in China for treating fever and malaria[2]. The active component of *qinghao* has been identified by Chinese scientists in the early 1970s and named *qinghaosu* (artemisinin). Although artemisinin and its analogs have initially been used for the treatment of malarial infection[3], their activity is not restricted to protozoans. Artemisinin has been well documented to possess anti-cancer activity against various tumor types, e.g. leukemia, melanoma and cancers of colon, lung, prostate, breast, ovarian, and central nervous system[4]. Most evidence shows that artemisinin and its analogs are relatively safe drugs with no obvious adverse reactions or noticeable side effects[5–9].

The most important pharmacological mechanism of artemisinin is that the artemisinin molecule contains an endoperoxide bridge, which can react with a ferrous iron atom in malaria parasites to form free radicals[10]. Generation of free radicals leads to macromolecular damages and cell death[11]. HCC cells have high rate of iron intake[12] and express a high cell surface concentration of transferrin receptors[13]. It is known that holotransferrin enhances the iron level of cancer cells by binding with transferrin receptors[14]. In this context, we hypothesized

Xiao-rong DENG, E-mail: dengxr77@163.com; Zhao-xia LIU, E-mail: lzxia77@163.com; Feng LIU, E-mail: washar1717@163.com
†These authors contributed equally to this work.
#Corresponding authors, Xiao-rong DENG, E-mail: dengxr77@163.com; He-ping YU, E-mail: yuheping12@163.com

that combination of holotransferrin and artemisinin may result in a synergistic effect on killing tumor cells. Hence, in this study, we investigated the combined effect of artemisinin and holotransferrin on human HCC SMMC-7721 cells *in vitro*. Emerging data show that artemisinin inhibits SMMC-7721 cells viability and proliferation, and causes SMMC-7721 cells apoptosis[15]. Interestingly, as we anticipated, holotransferrin significantly enhanced the anti-cancer activity of artemisinin.

1 MATERIALS AND METHODS

1.1 Materials and Cell Culture

All chemicals were purchased from Sigma (USA). TUNEL apoptosis detection kit (sc-4252) was obtained from Boehringer Mannheim (Germany). Human HCC cell line SMMC-7721 cells were obtained from ATCC (USA). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37ºC in a humidified incubator containing 5% CO_2.

1.2 Proliferation Assay

The effect of artemisinin and holotransferrin on growth of SMMC-7721 cells was measured by cell counting using a cytometer. SMMC-7721 cells seeded at a density of 2×10^3 cells per well to 6-well tissue culture plates in triplets were treated with artemisinin in the presence or absence of holotransferrin (1 μmol/L) at indicated concentrations. After incubation for 72 h, the cells were harvested by trypsination and the cell number was counted using a cytometor.

1.3 Colony Formation Assay

Colony formation assay in a soft agarose gel was performed to further determine the effects of artemisinin and holotransferrin on growth of SMMC-7721 cells. Briefly, 6-well tissue culture plates were coated with 1% agarose and the cells were plated at a density of 10^3 cells per well in triplets containing artemisinin in the presence or absence of holotransferrin. The cells were incubated at 37ºC in a humidified incubator containing 5% CO_2 for 2 weeks and then colonies ≥50 μm in diameter were counted.

1.4 Cell Viability Assay

The viability of SMMC-7721 cells treated with artemisinin and holotransferrin was assessed by trypan blue assay. Cells were seeded onto 6-well tissue culture plates at the density of 2×10^5 cells per well in DMEM supplemented with 10% FBS containing artemisinin at different concentrations with or without holotransferrin as indicated. DMEM was used as a vehicle. After cultured routinely for defined time periods, the cells were then detached by trypsinization and stained with trypan blue. The dead cells and the total cells were counted randomly three times. The cell viability was calculated by the ratio between viable cells and total cells. The cultured cells were then detached by trypsinization and stained with trypan blue. The dead cells and the total cells were counted. Viable cells (%) = [(Total cells−Dead cells)/Total cells]×100%.

1.5 Apoptosis Assay

Apoptosis of the SMMC-7721 cells treated with artemisinin and holotransferrin was measured by TdT-mediated dUTP nick end labeling (TUNEL) staining with an *in situ* cell death detection kit in accordance with the manufacturer's instructions. Briefly, SMMC-7721 cells were seeded on 6-well tissue culture plates (2×10^5 cells/well) in DMEM supplemented with 10% FBS containing artemisinin with or without holotransferrin. After incubation at 37ºC for 72 h, the apoptosis of SMMC-7721 cells was detected by using TUNEL. The number of apoptotic cells was counted randomly and the data were analyzed statistically by using SPSS 10.0. A value of $P<0.05$ was considered to be statistically significant.

Therefore, there were four treatment conditions: (1) cells treated with artemisinin (200 μmol/L) alone; (2) cells treated with holotransferrin (1 μmol/L) alone; (3) cells treated with both artemisinin and holotransferrin; and (4) cells without drug treatment (control).

2 RESULTS

2.1 Holotransferrin Enhances Growth Inhibitory Effect of Artemisinin against SMMC-7721 Cells

The effect of artemisinin in the presence or absence of holotransferrin on growth of SMMC-7721 cells was examined by using MTT assay. Treatment with artemisinin from 100–200 μmol/L resulted in significant inhibition of the proliferation of SMMC-7721 cells in a dose dependent manner (fig. 1). Interestingly, holotransferrin significantly enhanced the growth suppression of artemisinin against SMMC-7721 cells, suggesting that holotransferrin could sensitize the anti-growth effect elicited by artemisinin.

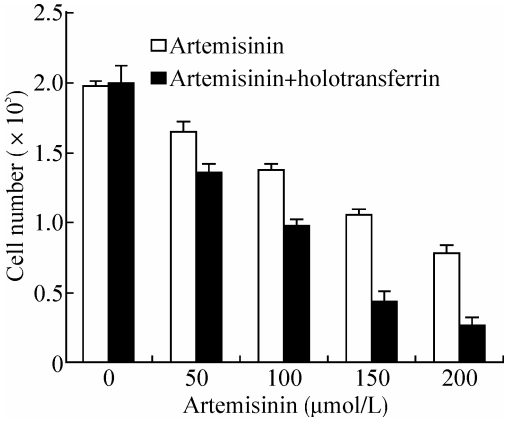

Fig. 1 Holotransferrin enhances the growth inhibitory effect of artemisinin against SMMC-7721 cells

SMMC-7721 cells were seeded on 96-well tissue culture plates at a density of 5×10^3 cells/well in DMEM supplemented with 10% FBS. Artemisinin at final concentrations of 0, 50, 100, 150 and 200 μmol/L with or without holotransferrin and the growth inhibitory effect was measured by MTT assay. Data are expressed as $x\pm s$ from a typical experiment. Proliferation of SMMC-7721 cells was significantly inhibited by artemisinin+holotransferrin as compared with artemisinin alone. ($P<0.01$).

2.2 Holotransferrin Enhances Inhibitory Effect of Artemisinin against Colony Formation of SMMC-7721 Cells

Transformed cells possess the property of forming tumors *in vivo* and forming colonies in soft agarose gel.

We employed this system to test the effect of artemisinin and holotransferrin on the colony formation. We observed that SMMC-7721 cells formed a great number of colonies after 2-week incubation with vehicle, whereas cells treated with artemisinin formed fewer colonies. Moreover, treatment with artemisinin plus holotransferrin completely inhibited the colony formation of SMMC-7721 cells, similar to their effect on cell growth (fig. 2).

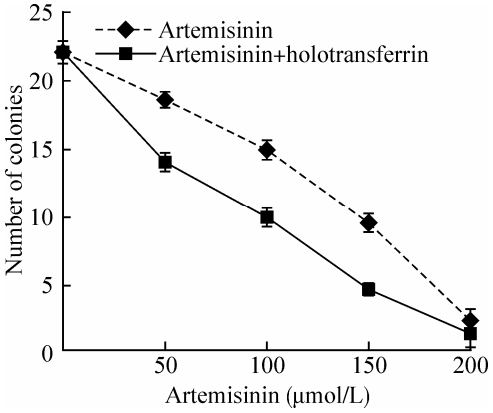

Fig. 2 Holotransferrin enhances the inhibitory effect of artemisinin against the colony formation of SMMC-7721 cells

SMMC-7721 cells were mixed in 0.4% agarose in DMEM supplemented with 10% FBS in the presence of artemisinin at concentrations of 0, 50, 100, 150, and 200 μmol/L with or without holotransferrin. After incubation for two weeks, colonies (50 μm in diameter) were counted randomly. Arteminin plus holotransferrin significantly inhibited the colony formation of SMMC-7721 cells as compared with artemisinin alone (P<0.01).

2.3 Holotransferrin Strengthens Effect on Tumor Cell Death Caused by Artemisinin

As holotransferrin could enhance the growth inhibitory effect of artemisinin against SMMC-7721 cells, we then set out to determine whether artemisinin and holotransferrin could kill the SMMC-7721 cells. To this end, we measured the cell viability using trypan blue staining assay. After incubation of SMMC-7721 cells with 50, 100, 150, 200 μmol/L artemisinin for 72 h respectively, the viability of the SMMC-7721 cells was 75.52%, 45.68%, 26.98%, and 8.56% respectively, whereas combination of holotransferrin with artemisinin significantly enhanced the cytotoxic effect caused by artemisinin, especially when the concentration of artemisinin ranged from 0–150 μmol/L (fig. 3).

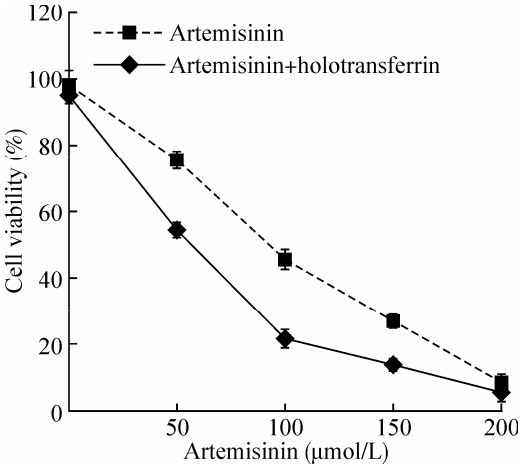

Fig. 3 The effect of artemisinin and holotransferrin on viability of SMMC-7721 cells

Data were expressed as the ratio between viable cells and total cells. Arteminin plus holotransterrin significantly induced cell death as compared with artemisinin alone (P<0.01).

2.4 Holotransferrin Enhances Artemisinin-induced Apoptosis of SMMC-7721 Cells

To investigate whether treatment with artemisinin and holotransferrin leads to apoptosis of SMMC-7721 cells, we examined apoptosis of SMMC-7721 cells after treatment with artemisinin and holotransferrin by using TUNEL assay. SMMC-7721 cells were seeded on 6-well tissue culture plates in DMEM supplemented with 10% FBS containing artemisinin with or without holotransferrin. After incubation for 24–72 h, we observed that artemisinin induced apoptosis of SMMC-7721 cells in a time- and dose-dependent manner. The number of the apoptotic cells in holotransferrin plus artemisinin group was significantly greater than that in artemisinin group (P< 0.01) (table 1).

Table 1 Holotransferrin enhances artemisini-induced apoptosis of SMMC-7721 cells

Groups	Apoptotic rate (%)		
	24 h	48 h	72 h
Control	0	0	0
Artemisinin (μmol/L)			
50	10.5±5.02	18.6±7.37	20.6±5.39
100	23.6±6.15	31.8±7.32	35.1±6.56
150	38.3±7.06	42.6±6.48	46.9±6.79
200	48.6±5.98	53.6±7.71	58.4±4.28
Artemisinin (μmol/L)+Holotransferrin			
50	59.3±3.39	64.8±4.59	68.5±3.36
100	68.6±5.96	71.6±5.13	73.2±4.50
150	74.6±9.82	77.6±6.94	80.6±4.35
200	79.6±5.83	85.6±8.33	90.6±6.67

The results of cells treated with holotransferrin (1 μmol/L) alone and those without drug treatment were not

shown.

3 DISCUSSION

HCC tissues have been demonstrated to possess a high cell surface concentration of transferrin receptors[13], which, after binding with holotransferrin, transport iron into the cells via a receptor-mediated endocytosis process[14]. It has been proven that the iron is a carcinogenic factor and is involved in facilitating the development of HCC[16]. Moreover, case control study implicates that hepatic iron overload may contribute to the development of HCC in cirrhotic or noncirrhotic patients[17]. Our primary data showed that artemisinin induced apoptosis of hepatoma SMMC-7721 cells depending on the concentrations of ferrous iron in cancer cells[18].

In this study, we observed that artemisinin inhibited the growth and colony formation of SMMC-7721 cells through inducing apoptosis. Of interest, we found that holotransferrin obviously enhanced the anti-cancer activity of artemisinin against SMMC-7721 cells. As mentioned above, incubation with holotransferrin could increase the concentration of ferrous iron in cancer cells[14]. The endoperoxide bridge in the artemisinin molecule could react with a ferrous iron atom and form free radicals, which enable artemisinin forming heme molecules with the large amount of iron[10]. Generation of free radicals leads to macromolecular damages and cell death[11]. This may imply that iron content in HCC can be increased, making the cells more susceptible to the cytotoxic effect of artemisinin-like compounds. Hence, artemisinin exhibited much stronger effect on killing mouse SMMC-7721 cells after combination with holotransferrin, likely due to increase the amount of iron in SMMC-7721 cells.

Normal organ development is controlled by a balance between cell proliferation and apoptosis[19]. In the case of cancer, the balance between proliferation and programmed cell death is disturbed with characteristics of uncontrolled proliferation and reduced apoptosis[20, 21]. Thus, induction of tumor cell apoptosis is an ideal way to kill cancer cells as well as inhibition of tumor cells proliferation. In this study, when treated with artemisinin and holotransferrin, SMMC-7721 cells underwent apoptotic death in the similar manner as reported by Singh[22]. Taken together, these preliminary results suggest that the anticancer effect of artemisinin and holotransferrin is caused by inhibition of tumor cell growth and induction of tumor cell apoptosis, thereby providing a promising therapeutic choice for HCC treatment, but the molecular mechanism needs further study.

Conflict of Interest Statement

We declare that we have no conflict of interest.

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